Preparation of fixative.

-Have 33 ml ddH_2O stirring in a 150 ml Erlenmeyer flask at 60^{o}C.
-Add 10µl of 10 M NaOH.
-Add 2 g of paraformaldehyde and stir until dissolved (few minutes). Do not over heat.
-Add 16.5 ml 3 x PBS buffer.
-Store on ice until use. The solution has to be used within 24 hours.

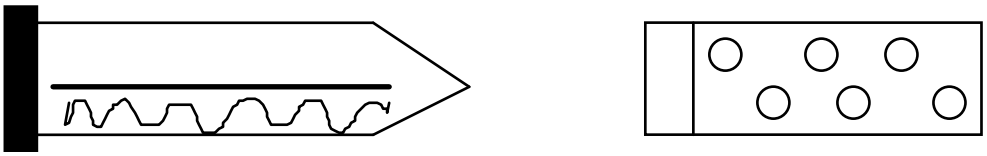
Fixation of Cell cultures.

-Harvest cells in exponential phase.
-Remove 1 ml of culture to a 1.7 ml eppendorf tube.
-Spin down cells at 14K for 3-5min.
-Remove 750 µl of supernatant.
-Resuspend cells (mix by vortexing) and add 750 µl fixative.
-Vortex for 1 min.
-Incubate cells at 4^{o}C for 5 min-24 hours.
-Spin down cells at 6K for 5 min and decant supernatant.
-Resuspend in 900 µl 1 x PBS buffer and add 100 µl 0.1 % NP40.
-Vortex for at least 1 min.
-Harvest the cells by centrifugation at 6K for 5 min.
-Add 500 ml 0.1% NP40 and resuspend cells by vortexing.
-Harvest cells by spinning 6K for 5 min.
-Resuspend in 200 µl 2 x storage buffer. Vortex for at least 1 min.
-Add equal volume of 96% EtOH and mix.
-Store cells at -20^{o}C.

NOTE: Fixed cells can be stored for months unless used for quantitative hybridizations. If the later is the case the cells have to be hybridized within a few weeks (50% degeneration in 2 months has been observed).

Hybridization.

The following protocol describes the use of formamide to regulate the stringency of the hybridization. The advantages of using formamide is that several hybridizations at different stringencies may be carried out simultaneously in the same incubator. Furthermore, the temperature can be kept at 37^{o}C which is not destructive to most biological material. A disadvantage of the formamide is that it slows down the annealing kinetics which have to be compensated for by prolonged hybridization times (do not try to compensate by increasing the probe concentration as this will raise the background).
Both the hybridization and the washing step are carried out in a humidified hybridization chamber (see Figure).



HYBRIDIZATION CHAMBER

A hybridization chamber is prepared as follows:

-Put a rolled-up 1M Wattman paper (or similar) into a 50 ml conical plastic tube.
-Wet the Wattman paper with approx. 2 ml buffer (20% formamide, 0.9 M NaCl, 0.1% SDS, 100 mM Tris pH 7.2).

NOTE: It is very important that the hybridization chamber is sealed well otherwise the wells will dry out.

Coated slides.

Most hybridizations are done on 6 or 8 wells heavy teflon coated slides. In order to immobilize the cells to the slide the slide must be coated e.g. gelatine or poly-L-lysin. **Coated slides will be available.**

The hybridization solution

The hybridization solution consists of:
20% formamide (FA), 0.9 M NaCl, 0.1% SDS, 100mM Tris pH 7.2. Since the formamide usually makes the solution more alkalic it is necessary to re-adjust the pH after mixing the hybridization solution (use conc HCl).
The typical concentration of formamide used is 10-40%. A rule of thumb is to hybridize at a stringency approximately equal to the probe-Td value (in this this course that will be 20% FA).This gives a signal of approximately half the maximum, and offers a good discrimination of nearly homologous target sequences. Some probes seem to work better when formamide is not included and the stringency is then regulated by salt/temperature.

Hybridization of cell cultures

-Apply 1-3 µl of fixed cells to a coated slide.
-Air dry.
-Dehydrate the cells in an ethanol series (50, 80, and 96%, 3 min. each). Do it in coplin jars.
-Air dry.
-Mix hybridization solution: volume = (number of wells used plus 1)*(9 µl of 15 % Formamide solution and 1 µl probe (25 ng)). **Keep dark !!**
- Apply 10 µl of hybridization solution to each well (make sure the solution cover the whole well and avoid scratching the surface).
- *NOTE: For quantitative hybridization it is important to mix the hybridization buffer and probe for all the wells and then subsequently apply the solution to each well. This ensures the same concentration of probe in all the wells.*

-Hybridize at 37^{o}C over night.

Washing procedure

-Rinse in ddH_2O
-Transfer the slide to a coplin jar with prewarmed (37^oC) washing solution I (=100 ml 20% FA solution).
-Incubate at 37^oC for 20 min.
-For DAPI staining, transfer the slide to a coplin jar containing the DAPI staining solution. Incubate for 5 min. at room temperature.
-Transfer the slide to 100 ml washing solution II (prewarmed, 37^oC) in a coplin jar and incubate for 15 min. at 37^oC.
-Rinse the slide by dipping into 100 ml ddH_2O at room temperature.
-Air dry in the dark and visualize.

Buffers and solutions:

TEAA buffer: 100 mM triethyl ammonium acetate.

3 x PBS buffer (390 mM NaCl in 30 mM $NaPO_4$ buffer adjusted to pH 7.2):

5 M NaCl	23.4 ml
0.5 M $NaPO_4$ buffer	18.0 ml
H_2O	258.6 ml

(0.5 M $NaPO_4$ buffer is prepared by mixing 28 ml 0.5 M NaH_2PO_4 with 72 ml 0.5 M Na_2HPO_4).

2 x storage buffer:
40 mM Tris pH 7.5, 0.2% NP40.

Washing solution II:

1 M Tris pH 7.2	10 ml
5 M NaCl	18 ml
H_2O	72 ml

DAPI staining solution:

1 M Tris pH 7.2	10 ml
5 M NaCl	18 ml
25 mg DAPI/ml	25 µl
H_2O	72 ml

Keep this solution in the dark. Is useful for about a week.

Microscopy.

It is important that the slide is *completely* dry before mounting the cells in oil.

-Cells hybridized with probes labelled with rhodamine, lissamine rhodamine B, CY3, or CY5 are mounted in Zeiss emission oil.

-Cells hybridized with fluorescein labeled probes are mounted in Citifluor.

-While using fluorescein with any other fluorochrome mount cells in Citifluor.

-X-gal assays are mounted in Citifluor.

Watch out! Take care not to mix emission oil and Citifluor.

Computer enhanced microscopy:

The exposure times are typically 500 msec and 5 sec with and without narrow band bypass filters, respectively. *Store all images named with your initials in your own directoryand remove files when copied to server.*

Filters in use:

	Filter set 1 + XF5	Filter set 10	Filter set 15	Filter set XF21	Filter set XF40	Filter set XF45+47
DAPI	X					
Fluorescein		X				
Rhodamine (TRITC)			X		X	
Acridine Orange (RNA)				X		
Lissamine rhodamine B			(X)		X	
CY 3			X		X	
CY 5						X

Data for the various filters:

				Excitation source	
	Exciter (nm)	Dichroic (nm)	Emitter (nm)	Hg	Xe
Filter set 1	BP 365/12	395	LP 397	X	
Filter set 10	BP 450-490	510	BP515-565	X	X
Filter set 15	BP 546/12	580	LP 590	X	X
Filter set XF05	BP 365/15	390	LP 400	X	
Filter set XF 21	BP 480/60	530	BP 635/55	X	X
Filter set XF 40	BP 560/40	590	LP 600	X	X
Filter set XF 45	BP 610/20	645	BP 670/40		X
Filter set XF 47	BP 640/20	670	BP 682/22		X

LP: Long pass; BP: Band pass.

Note that the Axioplan and the Axiovert are equipped with 100W mercury (HBO) and 75W xenon bulbs as excitation sources, respectively. Due to the mirror construction of the lamp

house of a Carl Zeiss microscope a 100W mercury bulb is useful only for 180 hours. *Furthermore, the light source must be fully aligned for quantitative purposes.*

NOTE: For all types of quantitative fluorescence microscopy it is necessary to counterstain the cells with DAPI and use this dye for focusing the CCD camera.

In addition, the Axioplan is equipped with a narrow band filter (590/10 nm) which can be used with filter set 14 and two narrow band by pass filters (546/12 nm and 530/10 nm) to be used with filter set 10. The Axiovert is equipped with the following narrow band filters (which also can be used on the Axioplan); BP500-530, SWP625, and BP670/20 to be used in combination with filters for fluorescein, rhodamine, lissamine rhodamine B, and CY5, respectively.

Image analysis

Following image analysis programs are currently in use:
DOS: PMIS v 2.11: Directly coupled to the CCD camera and used to control the camera.
Cellstat©: Automatically circumscribe each cell in the image. Quantifies accurately the light emitted from each cell (the fluorescent signal) and can give quantitative information about RNA, DNA and cell size for each cell.
Photoshop: Used to improve image quality for presentation. Reducing background noise using different image filters.

Flow cytometry
As a fast routine method flow cytometry can be used to determine cell size and DNA content in a large number of cells. Gives a statistical and exact measure for DNA and cell size distribution in a bacterial culture.

Fixation of cells.

- harvest 1 ml of cells (OD_{450} = 0,1 - 0,4) by spinning at 15000 rpm for 5 min.
- resuspend in 100 µl 10 mM Tris pH 7,5.
- add 1 ml 77 % ethanol
- Incubate cells at 4^{o}C for 5 min-24 hours

NOTE: Fixed cells can be stored for months.

DNA-Staining of cells.

- spin down 0,1 - 0,4 ml (depending at which OD_{450} they were harvested) at 15000 rpm for 10 min.
- remove all the supernatant with a gilson pipette .
- resuspend pellet in 200 µl Staining solution (keep dark!!).
- Cells are ready for flow cytometry after a few minutes.

Staining solution

90 µg /ml Mithramycin and 20 µg /ml EtBr dissolved in 10 mM Tris pH = 7,5, 10 mM $MgCl_2$.
This solution will be available on the course.
NOTE: Both Mitramycin and EtBr are highly carcinogenic compounds. Avoid to get in direct contact with skin. USE GLOVES !!.

About the Flow cytometer

The flow cytometer used in our laboratory is an Argus fluorescence based machine (Skatron, Norway)
The flow cytometer can simultaneously measure the forward light scatter (a measure for the cell size), and a eventual fluorescent signal emitted from the cell. In our case the fixed cells is stained with a DNA-specific fluorescent probe (Mithramycin). Thus, the fluorescent signal emitted from each cell will be proportional to the cellular DNA content. The DNA fluorescent signal is recorded using an Argus B1 filter block. System performance can be monitored by using 2 µm latex spheres. For each sample 20000 cells, using a flow rate of ~500 counts/µl, is analysed. The analysis accumulates as three dimensional (DNA/light scatter/cell number) histograms of the culture samples. Analysis of the flow cytometric histograms and transformation into two-dimensional presentations is performed by the use of the Windows based software “Winflow” developed by F.G.Hansen.